

Regulation of Sindbis Virus RNA Replication: Uncleaved P123 and nsP4 Function in Minus-Strand RNA Synthesis, whereas Cleaved Products from P123 Are Required for Efficient Plus-Strand RNA Synthesis

YUKIO SHIRAKO* AND JAMES H. STRAUSS

Division of Biology, California Institute of Technology, Pasadena, California 91125

Received 20 September 1993/Accepted 7 December 1993

Nonstructural proteins of Sindbis virus, nsP1, nsP2, nsP3, and nsP4, as well as intermediate polyproteins, are produced from two precursor polyproteins, P123 and P1234, by a proteolytic enzyme encoded in the C-terminal half of nsP2. We studied the requirements for and the functions of the intermediate and mature processing products for Sindbis virus RNA synthesis by using site-directed mutants which have a defect(s) in processing the 1/2, 2/3, or 3/4 cleavage sites either singly or in various combinations. A mutant defective in cleaving both the 1/2 and 2/3 sites, which makes only uncleavable P123 and mature nsP4 as final products, produced 10^{-3} as much virus as did the wild-type virus after 10 h at 30°C and was nonviable at 40°C. A mutant defective in processing the 2/3 site, which makes nsP1, nsP4, and P23 as well as precursor P123, grew 10^{-1} as efficiently as wild-type virus at 30°C and 10^{-3} as efficiently at 40°C. Early minus-strand RNA synthesis by these mutants was as efficient as that by wild-type virus, whereas plus-strand RNA synthesis was substantially decreased compared with that by wild-type virus. A mutant defective in processing the 3/4 site was nonviable at either 30 or 40°C. The 3/4 site mutant could be complemented by the mutant unable to cleave either the 1/2 or 2/3 site, which can provide mature nsP4. We interpret these results to signify that (i) mature nsP4 is required for RNA replication, (ii) nsP4 and uncleaved P123 function in minus-strand RNA synthesis, and (iii) cleavage of P123 is required for efficient plus-strand RNA synthesis. We propose that Sindbis virus RNA replication is regulated by differential proteolysis of P123. Early in infection, nsP4 and uncleaved P123 form transient minus-strand RNA replication complexes which vanish upon cleavage of P123. Later in infection, an elevated level of viral proteinase activity eliminates *de novo* synthesis of P123, and no further synthesis of minus-strand RNA is possible. In contrast, nsP4 and cleavage products from P123 form plus-strand RNA replication complexes which are stable and remain active throughout the infection cycle.

Sindbis virus is the prototype virus of the genus *Alphavirus* in the family *Togaviridae* (36). The virus genome is a plus-sense RNA of 11,703 nucleotides with a cap at the 5' end and a poly(A) tail at the 3' end (35). Four nonstructural proteins, nsP1, nsP2, nsP3, and nsP4, named in order of their position from the 5' end, are directly translated from the 5'-terminal two-thirds of the 49S genomic RNA, whereas three structural proteins, capsid, E2, and E1, are encoded in the 3'-terminal one-third and expressed from a 26S subgenomic RNA.

The four nonstructural proteins are initially translated as two overlapping polyproteins, P123 and P1234, the latter of which is produced by translational readthrough of an opal termination codon between nsP3 and nsP4. These polyproteins are cleaved into the four mature products, as well as into intermediate polyproteins, by a proteinase encoded in the C-terminal half of nsP2 (8, 15). It has been shown from *in vitro* translation studies that different nsP2-containing polyproteins have different activities in processing the three cleavage sites (6, 32). Notably, cleavage at the 1/2 site occurs in *cis* or in *trans* in P12 but only in *trans* in P123 or P1234; the 2/3 site in P123 and P1234 is not cleaved in *cis* but is cleaved rapidly in *trans* by P23; the 3/4 site in P1234 is cleaved efficiently in *cis* but is not cleaved efficiently in P34. As a result of these cleavage site

preferences, early in infection when viral protein concentration is low P123 and nsP4 are the major translation products, whereas late in infection when the protein concentration is high the mature proteins as well as P34 accumulate (6).

The functions of the four nonstructural proteins have been partially deduced from various studies. Amino acid sequence comparisons have shown that the N-terminal domain of nsP2 contains a nucleoside triphosphate (NTP)-binding helicase motif (10) and that nsP4 has an RNA polymerase motif (16). From genetic studies with temperature-sensitive mutants, it has been postulated that nsP1 is an initiation factor for minus-strand RNA synthesis (12, 28, 38), nsP2 is a factor for recognition of the 26S subgenomic promoter (12, 26), and nsP4 functions in template recognition and RNA elongation (2, 11, 24). nsP1 has also been shown to have activities suggesting that it is a capping enzyme, including methyltransferase and guanylttransferase activities (22, 31). nsP3 has also been shown to be required for RNA synthesis because mutations in it render the virus unable to synthesize RNA (4, 12), but no specific enzymatic activities have yet been ascribed to it. Until recently it was not known whether each protein functions only as the processed mature proteins or whether different polyprotein precursors also function in RNA synthesis. Preliminary studies implied that polyproteins P123 and P23 are functional in virus replication (32). Since nsP4 was made mostly early in infection whereas P34 accumulated late in infection, it was hypothesized that P34 might be a component of plus-strand RNA replication

* Corresponding author. Mailing address: Division of Biology 156-29, California Institute of Technology, Pasadena, CA 91125. Phone: (818) 395-4978. Fax: (818) 449-0756. Electronic mail address: shirako@iago.caltech.edu.

complex whereas nsP4 functions for both minus- and plus-strand RNA synthesis (37). More recently, it was demonstrated that uncleavable P123 plus cleavable P34 or N-terminally truncated forms of P34 can transcribe both minus- and plus-strand RNAs in BHK-21 cells, showing that polyproteins can function in RNA synthesis (20).

In this study we used a set of cleavage site mutants to investigate the requirements for and the functions of polyproteins and of mature proteins in Sindbis virus RNA synthesis. We found that uncleaved P123 and nsP4 function in minus-strand RNA synthesis but that cleaved products from P123 are required for efficient plus-strand RNA synthesis.

MATERIALS AND METHODS

Plasmid constructs, DNA preparation, and in vitro transcription. pToto1101, a full-length cDNA clone of Sindbis virus (23), was used as a wild-type (WT) parental clone to generate mutagenized derivatives. pToto1101.S has Ser in place of an opal termination codon at position 550 of nsP3 (7 amino acids upstream of the 3/4 cleavage site) (21, 32). Substitution of the Gly in the P2 position of the 1/2 and 2/3 cleavage sites by Val was described previously (32). The Gly (G_{GU}) in the P2 position of the 3/4 cleavage site was substituted with Val (G_{VU}) by site-directed mutagenesis (18); a *Bam*HI fragment (nucleotides 4634 to 7338, where nucleotide 1 is the first nucleotide in the Sindbis virus genome) from pToto1101 or pToto1101.S was cloned into bacteriophage M13 and the mutagenesis was carried out with a minus-sense mutagenic primer, 5'-AATATGTACCCA~~ACT~~ACCCC-3', annealing to nucleotides 5757 to 5776 (5a). The 3/4 site mutation, in either the pToto1101 or pToto1101.S background, was also combined with either or both of the 1/2 and 2/3 site mutations by replacing the *Bam*HI fragment of the 1/2 and 2/3 site mutants with that of the 3/4 site mutants. Thus, two sets of full-length cDNA clones containing one or combinations of the three cleavage site mutations were prepared in pToto1101 or pToto1101.S backgrounds. Mutants having a single amino acid substitution in the 1/2, 2/3, or 3/4 cleavage site were called mutants 1V, 2V, and 3V, respectively. Mutants having multiple cleavage site mutations were called mutants 12V, 123V, and so forth. The opal-to-Ser substitution was denoted by .S. A mutant containing Gly instead of Cys at position 481 of nsP2, the active site of the nsP2-proteinase (P2C481G) (34), was combined with an opal-to-Ser substitution to produce the mutant P2C481G.S. Miniprep DNA was prepared from transformed *Escherichia coli* MC1061.1 cells by a modified boiling method without RNase A treatment (32). RNA was transcribed in vitro from *Xho*I-linearized template DNA with SP6 RNA polymerase in the presence of a cap analog as described previously (23) with minor modifications.

In vitro translation of RNA transcripts. RNA transcripts from full-length cDNA clones in pToto1101 or pToto1101.S backgrounds were translated in rabbit reticulocyte lysates (Promega Biotec, Madison, Wis.), and the translated products were examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and fluorography as previously described (32).

RNA transfection, plaque assay, and virus growth analysis. RNA transcripts from cDNA clones in the pToto1101 background were used for transfection. Chicken embryo fibroblast monolayer cells were treated with 0.2 mg of DEAE-dextran per ml in Eagle's minimum essential medium (MEM) for 30 min and transfected for 30 min at room temperature with RNA transcripts diluted in phosphate-buffered saline (PBS). To examine plaque formation, transfected cells in 35-mm plates

were overlaid with 1% agarose in MEM containing 3% fetal calf serum, incubated at 30 or 40°C for 48 h, and stained with 5% Neutral red diluted in MEM. For virus growth analysis, transfected cells in 25-cm² flasks were incubated at 30 or 40°C in 5 ml of MEM containing 3% serum and samples of the medium were removed at intervals for viral plaque titer determination. To prepare virus stocks, the transfected cells were incubated in liquid medium at 30°C for 48 h and the medium was harvested.

Detection of Sindbis virus minus-strand RNA by PCR. Chicken monolayer cells in 25-cm² flasks (containing approximately 1.4×10^6 cells) were inoculated with WT virus or 2V mutant at a multiplicity of 10 or with 12V mutant at a multiplicity of 2. After incubation at 4°C for 1 h, the inoculum was removed, 5 ml of prewarmed medium was added, and the cells were incubated at 30 or 40°C for an appropriate period. The cells were trypsinized, suspended in cold PBS, and pelleted by centrifugation. They were then washed with cold PBS and lysed in 320 μ l of 0.3 M NaCl–10 mM Tris-HCl (pH 7.5)–1 mM EDTA–1% Nonidet P-40 with vigorous vortexing. The lysate was centrifuged at 12,000 rpm for 3 min to remove the nuclei, and the supernatant was mixed with 80 μ l of 10% SDS. The RNA was extracted twice with phenol-chloroform and once with chloroform, and it was precipitated with ethanol. The extracted cytoplasmic fraction was incubated in 10 μ g of DNase I (D4527; Sigma Chemical Co., St. Louis, Mo.) per ml in 0.3 M NaCl–50 mM Tris-HCl (pH 8.0)–10 mM MgCl₂ at 37°C for 30 min, and RNA was extracted with phenol-chloroform and precipitated in ethanol. The final pellet from WT virus- or 2V mutant-infected cells was resuspended in 70 μ l of water, whereas that from the 12V mutant-infected cells was suspended in 14 μ l of water. To 10 μ l of the DNase I-treated cytoplasmic extract was added 1 μ l of plus-sense primer 1 (200 pmol/ μ l) (5'-ATTGACGGCGTAGTACACAC-3' annealing to the 3'-terminal 20 nucleotides of the full-length minus-strand RNA). The mixture was heated at 100°C for 1 min and immediately cooled on ice. Another tube containing 25 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and 3 U of RNasin (Promega Biotec) in 10 μ l of 2 mM deoxynucleoside triphosphates (dNTPs)–0.1 M Tris-HCl (pH 8.35)–0.1 M KCl–50 mM dithiothreitol–16 mM MgCl₂ was also placed on ice. The contents of the two tubes were combined, immediately placed in a water bath at 42°C, and incubated for 30 min. One-tenth of the cDNA product was amplified by PCR as follows. cDNA product (2 μ l) was added to 98 μ l of 1 \times *Taq* buffer (10 mM Tris-HCl [pH 8.3], 1.5 mM MgCl₂, 50 mM KCl, 0.1 mg of gelatin per ml [Boehringer Mannheim Biochemicals]) containing 0.2 mM dNTPs and 40 pmol each of primer 1 and of primer 2 (5'-AAGTGTACGACTGCTTTC-3', which is a minus-sense primer annealing to nucleotides 865 to 882 of the viral genomic RNA). The mixture was heated at 100°C for 1 min and gradually cooled to room temperature over a period of 10 min. After addition of 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim Biochemicals) and overlay of 50 μ l of light mineral oil, the mixture was subjected to a thermal cycling reaction in a TwinBlock apparatus (Ericomp, San Diego, Calif.) as follows: one cycle at 56°C for 2 min and 72°C for 2 min; 24 cycles at 94°C for 10 s, 56°C for 30 s, and 72°C for 2 min; followed by an extension reaction at 72°C for 8 min. Then 2 μ l, i.e., 1/50 of the PCR product, originating from 4×10^2 cells infected with WT virus or the 2V mutant or from 2×10^3 cells infected with the 12V mutant, representing 4×10^3 PFU of inoculated virus, was electrophoresed in a 0.8% agarose gel in 50 mM Tris-acetate–1 mM EDTA (pH 8.0). DNA bands were visualized by staining with ethidium bromide. Lambda

phage DNA digested with *Hind*III was used for size and quantitation standards.

Preparation of dsRNA for a quantitative PCR control. Double-stranded RNA (dsRNA) corresponding to the 5'-terminal 5.9-kb region of Sindbis virus genomic RNA was prepared in vitro for a quantitative control in the PCR assay of minus-strand RNA. To obtain the 5'-terminal 5.9-kb plus-sense RNA strand, pToto1101 was linearized with *Kpn*I at nucleotide 5919, treated with T4 DNA polymerase in the presence of dNTPs, and transcribed with SP6 RNA polymerase. To obtain a 5.9-kb minus-sense RNA transcript with the exact 3'-terminal sequence, the 5'-terminal 5.9-kb region was cloned as follows: the 5'-terminal 2.1-kb region was amplified by PCR with a plus-sense primer, 5'-TGCTCTA GATTGACGGCGTAGTACACACTA-3', in which the bold-face hexamer is an *Xba*I site and the underlined sequence is nucleotides 1 to 22 of the Sindbis virus genome (strain HRsp), and a minus-sense primer, 5'-GTCAAACACGTACTCTG-3', complementary to nucleotides 2068 to 2084, with pToto1101 DNA as a template. The resulting 2.1-kbp DNA was digested with *Xba*I at nucleotide -5 and *Nsi*I at nucleotide 2001, and the 2.0-kbp *Xba*I-*Nsi*I fragment was gel purified. This *Xba*I-*Nsi*I fragment was cloned in a three-piece ligation with an *Nsi*I-*Eco*RI fragment from pToto1101 (nucleotides 2001 to 5869), into *Eco*RI-*Xba*I-digested pGEM4Z (Promega Biotec). The cloned DNA was linearized with *Xba*I, and the 5'-terminal 5.9-kb minus-sense RNA was transcribed with SP6 RNA polymerase in vitro. Equal amounts of the plus-sense and minus-sense transcripts were combined directly after the transcription reaction, heated at 70°C for 1 min, and annealed by gradually cooling to room temperature. After incubation with DNase I at 37°C for 30 min to remove template DNA, the dsRNA was extracted with phenol-chloroform, precipitated with ethanol, and run in an LMP agarose gel. The distinct 5.9-kb dsRNA was purified from a sliced gel by a modified cetyltrimethylammonium bromide (CTAB) method, resuspended in water, and serially diluted from 300 pg/μl (5×10^7 molecules per μl) to 3 fg/μl (5×10^2 molecules per μl) in water or in extracts from uninfected chicken cells. A 1-μl sample of dsRNA at each dilution was used for cDNA synthesis in a total volume of 20 μl; this was followed by PCR amplification as described above for cytoplasmic RNA extracts from virus-infected cells. Then 1/50 of the PCR product, originating from 10^5 molecules to 1 molecule of dsRNA, was electrophoresed in a 0.8% agarose gel.

Accumulation of RNA in infected cells. Chicken cells in 35-mm plates (approximately 5×10^6 cells) were infected with WT virus, with the 2V mutant, or with the 12V mutant as described for the PCR assay of minus-strand synthesis. After adsorption of the virus to the cells in PBS at 4°C, the cells were incubated in Eagle's MEM containing 3% fetal calf serum and 5 μg dactinomycin per ml at 30 or 40°C for 2 h. The medium was then replaced with Eagle's MEM containing 3% serum and 20 μCi of [³H]uridine per ml (37.5 Ci/mmol; DuPont/NEN, Boston, Mass.), and the infected cells were incubated for an additional 1, 5, or 10 h at the same temperature. The cells were lysed, and total RNA was isolated with RNeasy (TEL-TEST, Friendswood, Tex.) by the method recommended by the manufacturer. Two-fifths of the extracted RNA was spotted onto DE81 filter paper, the filter was washed in 0.5 M Na₂HPO₄, and radioactivity was assayed by liquid scintillation counting.

For Northern (RNA) blot analysis of plus-strand RNA accumulation, chicken cells were infected, incubated at 40°C, and harvested as described above. After being treated with trypsin and washed in cold PBS, infected cells were lysed in 320

μl of 0.1 M glycine (pH 9.5)-0.1 M NaCl-10 mM EDTA-1% Nonidet P-40 on ice. The cytoplasmic RNA was extracted by an SDS-phenol method and precipitated in ethanol, and the pellet was resuspended in water. Approximately 2 μg of total cytoplasmic RNA was denatured in 1.1 M formaldehyde-45 mM Tris-borate-1 mM EDTA at 65°C for 5 min and run in a 0.8% agarose gel. RNA was capillary transferred to non-charged nylon membrane (Hybond-N; Amersham International, Amersham, United Kingdom) by using 3 M NaCl-0.3 M sodium citrate for 3 h. The membrane was baked at 80°C for 1 h and incubated in 50% formamide-5% polyethylene glycol-7% SDS-0.25 M sodium phosphate (pH 7.2)-0.25 M NaCl-1 mM EDTA at 56°C for 30 min. To prepare ³²P-labeled minus-sense RNA probe, an *Stu*I-*Sal*I fragment from pToto1101 (nucleotides 8571 to 10770) was cloned into *Sma*I-*Sal*I-digested pGEM3Z. Minus-sense riboprobe was transcribed from *Eco*RI-digested DNA with SP6 RNA polymerase in the presence of [³²P]CTP (3,000 Ci/mmol; Du Pont/NEN). After addition of ³²P-labeled RNA probe to the hybridization buffer, the membrane was incubated for 8 h at 56°C and washed twice in 2% SDS-0.25 M sodium phosphate (pH 7.2)-1 mM EDTA at 56°C for 30 min and twice in 1% SDS-40 mM sodium phosphate (pH 7.2)-1 mM EDTA at 65°C for 30 min. The membrane was air dried and exposed to an X-ray film.

RNA electroporation and immunofluorescence assay. Nearly confluent monolayers of BHK-21 cells were trypsinized, washed, and suspended in PBS at 10^7 cells per ml. Approximately 20 μg of RNA transcripts synthesized in vitro was added to 0.4 ml of the cell suspension in an electroporation cuvette (0.2-mm electrode distance; Bio-Rad, Richmond, Calif.), and the suspension was subjected to two electric pulses of 1,500 V by using a Bio-Rad Gene Pulser electroporator. For the immunofluorescence assay, the electroporated cells were suspended in 10 ml of MEM containing 10% fetal calf serum, glutamine, and nonessential amino acids. Then 100 μl of the diluted cell suspension was incubated in wells of a 96-well multititer plate at 40°C for 15 h. The cells were fixed with methanol, treated with a mixture of anti-E1 and anti-E2 rabbit polyclonal sera diluted 1/100 in PBS as primary antibodies and then with goat anti-rabbit immunoglobulin G conjugated to fluorescein isothiocyanate (F0382; Sigma Chemical Co.), and observed with a fluorescence microscope.

RESULTS

Cell-free translation products produced from in vitro transcripts of cleavage site mutants. Previously we showed that substitution of the penultimate Gly in the 1/2 or 2/3 cleavage sites with either Val or Glu abolished the processing at the mutated site, that abolition of processing at the 1/2 site also prevented efficient processing at the 2/3 site but abolition of cleavage at the 2/3 site did not interfere with processing at the 1/2 site, and that in either case the 3/4 site was cleaved efficiently (32). For the current studies, the effect of substitution of the penultimate Gly in the 3/4 cleavage site upon processing at the three cleavage sites was also examined. Although the work in infected cells reported in this paper involves only viruses with the WT opal codon just upstream of the N terminus of nsP4, such that readthrough of this codon is required to produce polyproteins containing nsP4, we also included constructs containing a serine codon in place of the opal codon in in vitro translation experiments. Because readthrough of the opal codon is inefficient, the serine-containing constructs allowed a more accurate assessment of the extent of processing at the 3/4 site and allowed us to estimate the efficiency of readthrough at 30 and 40°C.

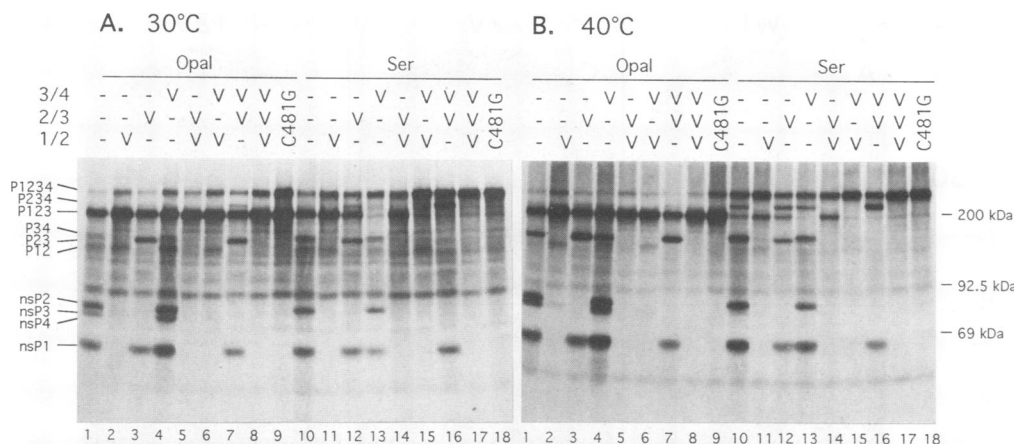


FIG. 1. In vitro translation and processing products in rabbit reticulocyte lysates directed by RNA transcripts from full-length cDNA clones of Sindbis virus nonstructural processing mutants. (A) Incubation at 30°C for 90 min. (B) Incubation at 40°C for 90 min. Lanes: 1, WT; 2, 1V; 3, 2V; 4, 3V; 5, 12V; 6, 13V; 7, 23V; 8, 123V; 9, P2C481G; 10, WT.S; 11, 1V.S; 12, 2V.S; 13, 3V.S; 14, 12V.S; 15, 13V.S; 16, 23V.S; 17, 123V.S; 18, P2C481G.S. Translated products were run on SDS-PAGE (7.5% polyacrylamide) and detected by fluorography (5, 32).

Figure 1 shows proteins produced at 30°C (Fig. 1A) and at 40°C (Fig. 1B) upon translation in cell-free extracts of transcripts from the mutated constructs. In polyproteins translated from transcripts from 1V and 1V.S mutants (lanes 2 and 11), cleavage at the 1/2 site was abolished, cleavage at the 2/3 site occurred inefficiently, and cleavage at the 3/4 site occurred as efficiently as in the WT strain. As a result, P123 and P1234 were the major products, with minute amounts of P12 and nsP3 in the 1V mutant and of P12 and P34 in the 1V.S mutant. Cleavage at the 3/4 site occurred efficiently in both 1V and 1V.S mutants, but mature nsP4 is difficult to detect even in the 1V.S mutant owing to its rapid degradation by an N-end rule pathway (7). Translation of 2V and 2V.S transcripts resulted in the synthesis of P1234, P123, P234, P23, and nsP1, indicating that cleavage at the 2/3 site was blocked but the 1/2 and 3/4 sites were cleaved efficiently (lanes 3 and 12). Translation products from WT and 3V transcripts were indistinguishable (lanes 1 and 4), except for larger amounts of P1234 in 3V at 30°C. However, there was no P123 in the translation products from 3V.S transcripts (lanes 13), indicating that cleavage at the 3/4 site was abolished but the 1/2 and 2/3 sites were cleaved in both 3V and 3V.S mutants. A Cys-to-Gly substitution at position 481 in nsP2 abolished processing at all three sites as reported previously (34), and only P1234 and P123 were made from P2C481G mutant (lanes 9) and only P1234 was made from P2C481G.S mutant (lanes 18).

There were differences in the efficiencies of processing at 30 and 40°C. Cleavage at the 1/2 and 2/3 sites appeared to occur more efficiently at 40°C than at 30°C as judged from the accumulation of nsP1, nsP2, and P34 in the WT.S and 3V.S translation products (Fig. 1, lanes 10 and 13). In contrast, cleavage at the 3/4 site appeared less efficient at 40°C than at 30°C as judged from the accumulation of P34 in the WT and WT.S translation products (lanes 1 and 10) and a greater P1234/P123 ratio in the 12V.S translation products at 40°C than at 30°C (lanes 14). The accumulation of P234 in the WT.S and 3V.S translation products at 40°C indicated that the 2/3 site in P234 could not be cleaved efficiently at the higher temperature (lanes 10, 12, and 13). In addition, similar amounts of P123 and P234 were produced from 2V.S transcripts at 40°C, but much less P234 than P123 was produced at 30°C (lanes 12), indicating that at 40°C the 1/2 and the 3/4 sites were cleaved with an equal efficiency, whereas at 30°C the 3/4

site was more efficiently cleaved than at the 1/2 site. These differences in processing efficiencies may be due to changes in proteinase activities, to altered substrate structures, or to changes in the kinetics of translation at the two temperatures.

There was also a difference in the efficiency of readthrough of the opal termination codon near the end of nsP3 at the two temperatures. At 30°C, the opal codon was read through with about 20% efficiency as reported by de Groot et al. (6), whereas at 40°C the readthrough appeared to occur with less than 5% efficiency, as judged from the P1234/P123 ratios in the translation products from the 123V and P2C481G transcripts (lanes 8 and 9).

Growth of mutant viruses. After transfection of chicken cells with WT transcripts, large plaques were formed at both 30°C (Fig. 2A) and 40°C (Fig. 2B). The 2V mutant formed small plaques at 30°C (Fig. 2C) and minute plaques at 40°C (Fig. 2D). The 1V mutant (data not shown) and the 12V mutant formed minute plaques at 30°C (Fig. 2E) but no plaques at 40°C (Fig. 2F). In contrast, none of the 3/4 site mutants nor the P2C481G mutant formed any plaques at either temperature. Approximately 2×10^3 to 5×10^3 plaques were consistently formed per μg of RNA transcripts from the WT or from the viable mutant constructs, indicating that the viability of the mutants does not result from the presence of revertants or suppressor mutations in the virus.

To further study the growth of the mutants, we incubated transfected cells at 30 or 40°C in liquid medium. The titer of the virus released into the medium was determined (Fig. 3A and 3B), and the cells were examined for the appearance of cytopathic effects. As judged from the number of plaques formed directly after RNA transfection, fewer than 1% of the cells appeared to be initially transfected when DEAE-dextran was used. However, at 30°C most of the WT-transfected cells developed cytopathic effects within 24 h and the cells transfected with the 2V or 12V mutants developed cytopathic effects within 48 h, owing to secondary infection. WT virus started to accumulate in the medium after 5 h at 40°C or 7 h at 30°C. Release of the 2V virus was delayed by 2 h compared with WT virus at both temperatures. Release of the 12V virus was delayed by 5 h compared with WT virus at 30°C, but no virus was produced at 40°C, confirming that the 12V mutations are lethal in terms of virus production at 40°C. The 1V mutant gave a growth curve similar to that of the 12V mutant but

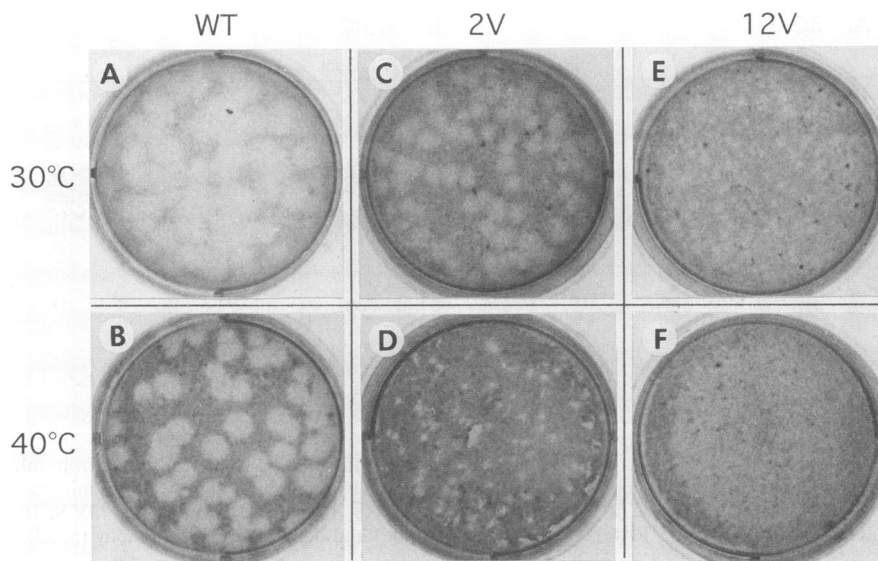


FIG. 2. Plaque formation on chicken monolayer cells after RNA transfection. Chicken cells were transfected with WT transcripts (A and B), the 2V transcripts (C and D), or the 12V transcripts (E and F). After transfection, the cells were incubated at 30°C (panels A, C, and E) or 40°C (panels B, D, and F) for 48 h before being stained.

occasionally produced variants forming large plaques when incubated for prolonged periods, owing to reversion of the mutated Val in the 1/2 cleavage site to Gly or Ala (unpublished results). The virus titer after 48 h of incubation at 30°C was more than 10^9 PFU/ml after WT or 2V transfection and around 10^6 PFU/ml after 12V transfection. Thus for the WT virus, the 2V mutant, and the 12V mutant, there was a correlation between virus growth and plaque size at both temperatures.

Rescued viruses containing the 2V or 12V mutation formed the same-sized plaques as those formed directly after RNA transfection. Virus growth curves obtained from these viruses were essentially the same as those after RNA transfection except for the shorter latent periods (Fig. 3C and D). WT virus was released into medium beginning at 3 and 5 h postinfection at 40 and 30°C, respectively. Release of 2V virus was delayed by 2 h compared with release of WT virus at either temperature. Release of 12V virus was delayed by 5 h compared with release of WT virus at 30°C and was not detected at 40°C, confirming that the 12V mutations are lethal at 40°C.

None of the cells transfected with the 3/4 site transcripts or the P2C481G transcripts released viable viruses into the media or developed any significant cytopathic effects at either temperature, even after 5 days of incubation, indicating that these mutations are lethal.

To confirm that the lethality of the 3V mutant results from the substitution of the penultimate Gly in the 3/4 cleavage site with Val, the *Bam*HI fragment (nucleotides 4634 to 7338), which was cloned from M13 replicative-form dsDNA after in vitro mutagenesis, was entirely sequenced and the sequence was compared with that of pToto1101. The 3V mutant had the expected change at nucleotide 5764 (from G_{pToto1101} to T), leading to substitution of the P2 amino acid of the 3/4 site (from Gly_{pToto1101} to Val). No other changes were found between the two sequences. To further show that no outside change was responsible for the mutant phenotype, the *Bam*HI fragment of the 3V mutant was replaced with the corresponding fragment from pToto1101. When in vitro transcripts from

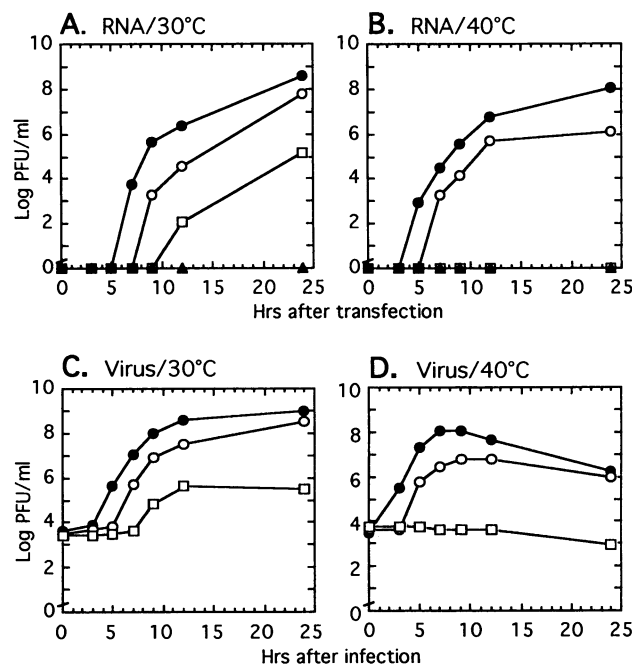


FIG. 3. Growth curve analysis of WT virus, the 2V mutant, the 12V mutant, and the 3V mutant. (A) Incubation at 30°C after RNA transfection. (B) Incubation at 40°C after RNA transfection. (C) Incubation at 30°C after virus infection. (D) Incubation at 40°C after virus infection. Symbols: ●, WT transcripts or virus; ○, the 2V transcripts or virus; □, the 12V transcripts or virus; ▲, the 3V transcripts. For determination of plaque titer, chicken cells were inoculated with serially diluted virus samples and incubated at 30°C for 48 h before being stained.

TABLE 1. Proteolytic processing products and viability of Sindbis virus nonstructural processing mutants

Construct	Presence of proteolytic processing product										Viability/plaque size ^c at:	
	nsP1	nsP2	nsP3 ^a	nsP4 ^b	P12	P23 ^a	P34	P123 ^a	P234	P1234	30°C	40°C
WT	+	+	+	+	+	+	+	+	+	+	+ /large	+ /large
1V	–	–	(+) ^d	+	(+)	–	(+)	+	–	+	+ /minute	–
2V	+	–	–	+	–	+	–	+	+	+	+ /small	+ /minute
3V	+	+	+	–	+	+	+	+	+	+	–	–
12V	–	–	–	+	–	–	–	+	–	+	+ /minute	–
13V	–	–	(+)	–	(+)	–	(+)	+	–	+	–	–
23V	+	–	–	–	–	+	–	+	+	+	–	–
123V	–	–	–	–	–	–	–	+	–	+	–	–
P2C481G	–	–	–	–	–	–	–	+	–	+	–	–

^a There are two forms of nsP3 and nsP3-containing polyproteins; one is produced by cleavage at the 3/4 site, and the other is terminated at an opal termination codon 7 amino acids upstream of the 3/4 cleavage site. When the 3/4 site is not cleavable, only the latter forms are produced.

^b The mature nsP4 is difficult to detect in *in vitro* translation products owing to down-regulation by an in-frame, partial termination codon located upstream of the nsP4 gene and to rapid degradation by the N-end rule pathway (7).

^c Plaque sizes were compared after 48 h of incubation. A minus sign indicates that no plaques were formed and no cytopathic effects were observed after RNA transfection and that no virus was detected in the media.

^d When cleavage at the 1/2 site is abolished, cleavage at the 2/3 site occurs at a very low efficiency and only trace amounts of certain products were produced (+).

this construct were transfected into chicken cells, only large plaques were formed at 30 and 40°C. Therefore, the regions outside of the *Bam*HI fragment do not have mutations which might have conferred the lethality of the 3V mutant, confirming that the substitution of Gly at the P2 position of the 3/4 site with Val is lethal.

Table 1 summarizes the translation products from and the viability of each mutant.

Early minus-strand RNA synthesis by WT virus or by the 2V mutant. Since the 2V and 12V mutants cannot complete the normal processing pathway of the nonstructural proteins, which are components of the RNA replication complexes, their reduced and delayed growth, temperature sensitivity, and lethality are probably due to a defect(s) in viral RNA synthesis. To ascertain which step(s) of the RNA synthesis is impaired in the two mutants, the initial step of the virus RNA replication, the synthesis of minus-strand RNA, was analyzed by a PCR method.

From alphavirus-infected cells, minus-strand RNA is isolated as a double-stranded form, either because the minus-strand RNA is double stranded in the cell, forming replicative intermediates with plus-strand RNA, or because the minus-strand RNA hybridizes with plus-strand RNA present in large molar excess during the isolation procedure. To assess the detection limit of minus-strand RNA, dsRNA representing the 5'-terminal 5.9-kb region was produced *in vitro*. In ethidium bromide-stained agarose gels, a 0.9-kb PCR product originating from 10² molecules of the dsRNA was detectable whether the dsRNA was suspended in water (Fig. 4A) or in extracts from uninfected chicken cells (Fig. 4B). The 0.9-kb PCR product was not detected without first-strand cDNA synthesis (data not shown). Digestion of the 0.9-kb PCR product with *Hind*III generated two fragments of 0.12 and 0.76 kb (data not shown), proving that the 0.9-kb DNA was amplified from cDNA corresponding to the 3'-terminal region of the minus-strand RNA. The amount of PCR product increased with increasing numbers of dsRNA molecules up to 10³ to 10⁴ and then plateaued, indicating that quantitative comparison of minus-strand RNA was feasible within the range of more than 10 to less than 10⁴ molecules by this PCR method.

To examine early minus-strand RNA synthesis, chicken cells were inoculated with WT virus or the 2V mutant at a multiplicity of 10. Infection was synchronized by keeping the cells at 4°C during the inoculation period and then shifting the tem-

perature to 30 or 40°C and incubating the infected cells for up to 24 h. Minus-strand RNA was assayed at intervals. In WT virus-infected cells, minus-strand RNA was not detectable before incubation at 30 or 40°C or within the first 20 min after the temperature was raised. Distinct 0.9-kb DNA appeared at 30 min after incubation at either 30°C (data not shown) or 40°C (Fig. 4C). This indicates that minus-strand RNA was not present or was present in amounts below the detection limit during the first 20 min of incubation and that the minus-strand RNA detectable at 30 min was synthesized *de novo* after virus infection was established at the higher temperature. The amount of minus-strand RNA continued to increase during the first 3 h of incubation (Fig. 4C) and then appeared to remain constant for up to 24 h at either 30 or 40°C (Fig. 4E). In cells infected with the 2V mutant and incubated at either 30 or 40°C, minus-strand RNA was also detectable at 30 min postinfection (Fig. 4D); the amount increased significantly up to 3 h postinfection (Fig. 4D and F) and then remained constant (at 30°C) or declined (at 40°C) with long incubation (Fig. 4D and F). These results indicate that minus-strand RNA synthesis during the early stages of infection is indistinguishable in WT virus-infected and 2V mutant-infected cells.

Minus-strand RNA synthesis in the 12V mutant-infected cells. Chicken cells were infected with the 12V virus at a relatively low multiplicity of 2 because it was difficult to obtain virus stocks with a titer greater than 10⁷ PFU/ml. Therefore, cytoplasmic extracts were concentrated fivefold compared with those from WT virus- or 2V mutant-infected cells so that the numbers of viral RNA molecules would be comparable. We found that when different multiplicities of infection with WT virus were used, the amount of minus-strand RNA synthesized early after infection was proportional to the multiplicity, and therefore the use of increased amounts of 12V-infected extract is expected to compensate for the lower multiplicity.

At 30°C, minus-strand RNA was detectable at 30 min after infection with 12V (Fig. 4G). The amount of minus-strand RNA continued to increase during a 12-h incubation period. When the cells were incubated at 40°C, minus-strand RNA was also detectable at 30 min after infection (Fig. 4H). The amount of minus-strand RNA gradually increased up to 2 h after infection but decreased significantly after 3 h. The maximum number of minus-strand RNA molecules synthesized at 40°C (from 4 × 10³ PFU of the input virus) appeared to be nearly 10³ (this result was obtained by comparing the PCR products

with those from serially diluted 5.9-kbp dsRNA), indicating that a significant fraction of the input 12V mutant RNA could be transcribed into minus-strand RNA. When the cells were incubated for 24 h at 30, 37, or 40°C, minus-strand RNA was clearly detectable after incubation at 30°C, barely detectable after incubation at 37°C, and not detectable after incubation at 40°C (Fig. 4I). The virus titer in the media at 24 h after infection was 5×10^5 PFU/ml at 30°C, 8×10^3 PFU/ml at 37°C, and 2×10^2 PFU/ml at 40°C. These results indicate that

initial minus-strand RNA synthesis by the 12V mutant was as efficient as that by WT virus or by the 2V mutant but that subsequent plus-strand RNA synthesis was severely impaired or did not occur at 40°C. The minus-strand RNA transcribed from the input viral RNA was probably degraded during the 24-h incubation at 40°C.

Late plus-strand RNA synthesis. Plus-strand RNA synthesis after infection by WT virus, the 2V mutant, or the 12V mutant was assayed by incubating infected cells in the presence of [³H]uridine and determining the amount of label incorporated into RNA. More than 90% of RNA synthesized in alphavirus-infected cells is plus-strand RNA, and thus the incorporation of label is essentially a measure of plus-strand RNA synthesis. As shown in Fig. 5A, WT- and 2V mutant-infected cells incorporated the same amount of label at 30°C. Mutant 12V-infected cells incorporated an amount of label that was just above the background level incorporated by uninfected cells, demonstrating that plus-strand RNA synthesis is severely impaired in this mutant, as predicted from the results with minus-strand RNA synthesis. The incorporation at 40°C is shown in Fig. 5B. The 2V mutant also exhibited a defect in plus-strand RNA synthesis at this temperature, incorporating less label than the WT virus did. The 12V mutant incorporated no detectable uridine above background at 40°C, showing that

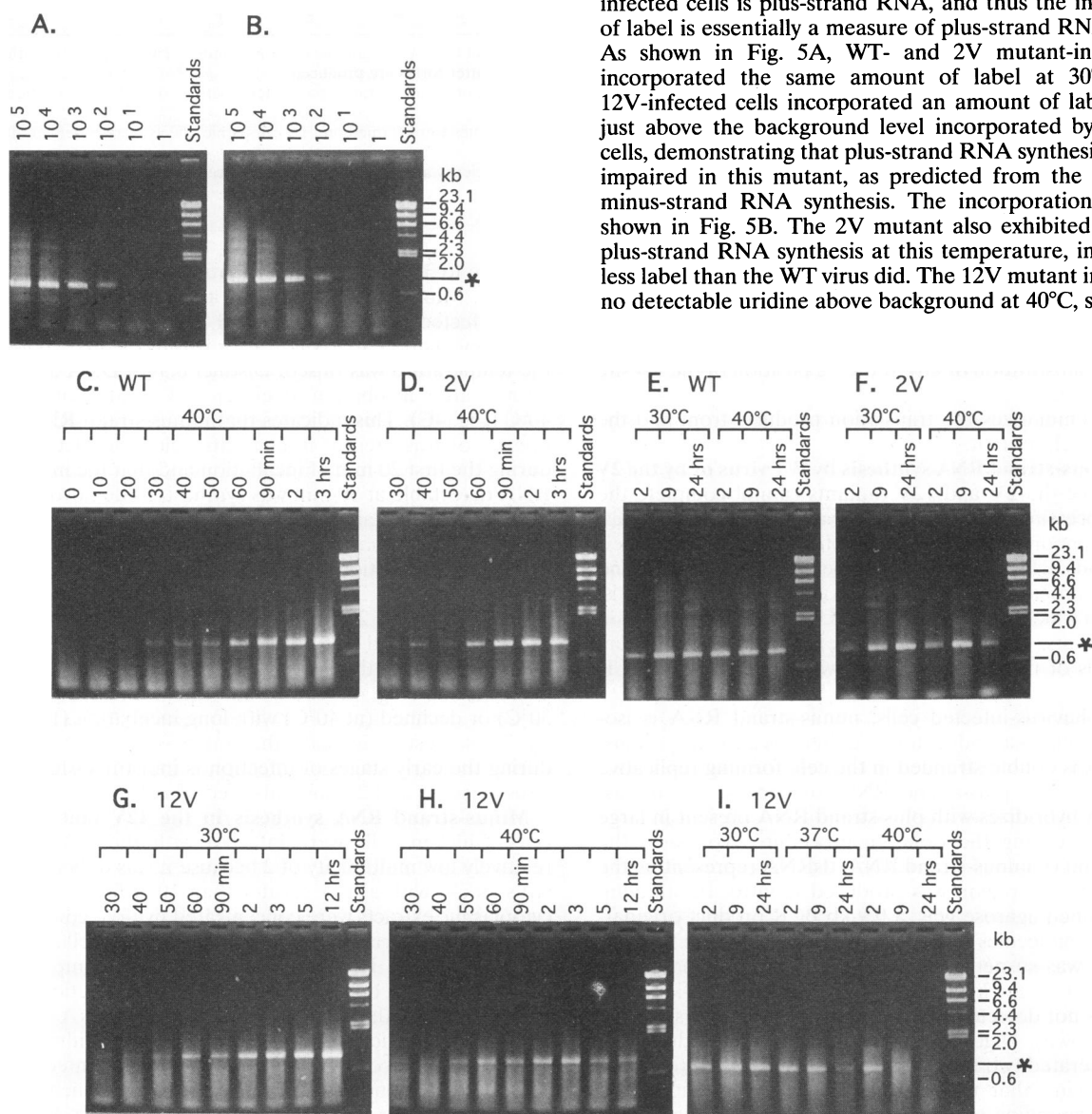


FIG. 4. Detection of minus-strand RNA by PCR. (A and B) dsRNA was prepared by hybridizing plus-sense and minus-sense in vitro transcripts representing the 5'-terminal 5.9-kb region. The dsRNA was serially diluted in water (panel A) or in extracts from uninfected chicken cells (panel B) before cDNA synthesis and amplification of 0.9 kb in the 3'-terminal region of the minus-strand RNA. The number of dsRNA molecules used to generate the PCR product loaded in each lane is shown at the top. (C to I) cDNA was synthesized to minus-strand RNA in cytoplasmic extracts from WT virus-infected chicken cells incubated at 40°C for up to 3 h (panel C), 2V mutant-infected cells incubated at 40°C for up to 3 h (panel D), WT virus-infected cells incubated at 30 or 40°C for up to 24 h (panel E), 2V mutant-infected cells incubated at 30 or 40°C for up to 24 h (panel F), 12V mutant-infected cells incubated at 30°C for up to 12 h (panel G), 12V mutant-infected cells incubated at 40°C for up to 12 h (panel H), or 12V mutant-infected cells incubated at 30, 37, or 40°C for up to 24 h (panel I). Incubation periods are shown above the gels. Lambda phage DNA digested with *Hind*III (100 ng in total per lane) was used for size and quantitation standards.

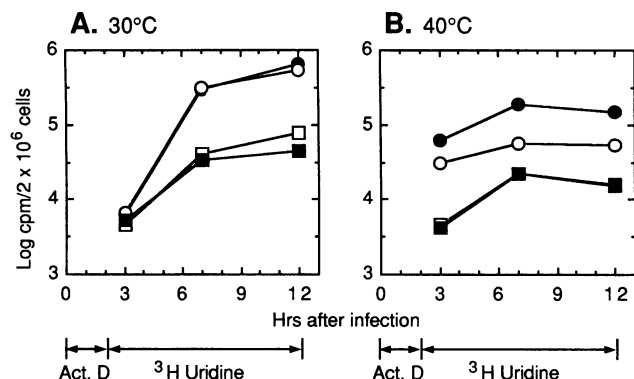


FIG. 5. Analysis of RNA synthesis in chicken cells after infection with WT virus, the 2V mutant, or the 12V mutant at 30°C (A) or 40°C (B). Monolayers of chicken cells were infected with the WT virus (●) or the 2V mutant (○) at a multiplicity of 10 or with the 12V mutant (□) at a multiplicity of 2 or mock infected (■) at 4°C for 1 h. The cells were then shifted to 30 or 40°C in the presence of dactinomycin (Act. D) for 2 h. The medium was then replaced with [³H]uridine-containing medium, and the infected cells were incubated for an additional 1, 5, or 10 h at 30 or 40°C. The cells were then harvested and assayed for the amount of ³H incorporated into RNA.

no detectable plus-strand RNA synthesis occurred following infection at 40°C.

Plus-strand RNA accumulation at 40°C by WT virus and by the 2V mutant were also compared by Northern blotting of cytoplasmic RNA isolated at 0, 3, 5, and 7 h postinfection (Fig. 6). At 3 h after infection with the 2V mutant, genomic and subgenomic RNAs were readily detected but the amounts were less than 10% of that produced in cells infected by WT virus. Amounts of both RNA species increased during the next 2 h, but after 5 h of infection a significant increase was not observed in the 2V mutant-infected cells. In contrast, in WT virus-infected cells genomic and subgenomic RNA levels continued to increase throughout the 7-h incubation period. For both viruses, the molar ratios of genomic to subgenomic RNAs appeared to be less than 1:20, probably owing to inefficient transfer of larger RNA molecules during blotting. The results are consistent with the uridine incorporation assays.

Complementation of the 3V mutant. To determine whether the 3V mutant could be rescued by supplying mature nsP4, we

carried out a complementation experiment between the 3V mutant and the 12V mutant. BHK-21 cells were transfected with in vitro transcripts of WT virus, the 3V mutant, the 12V mutant, or a mixture of the 3V and the 12V transcripts by using electroporation. After incubation at 40°C for 15 h, cells were examined by an immunofluorescence assay with a mixture of anti-E1 and anti-E2 antisera to detect viral glycoproteins produced in the transfected cells. When the cells were transfected with WT transcripts, more than 50% of the cells fluoresced intensely (Fig. 7A). When they were transfected with the 3V transcripts (Fig. 7B) or the 12V transcripts (Fig. 7C), the fluorescence was essentially the same as that seen with nontransfected control cells. However, when a mixture of the 3V transcripts and the 12V transcripts were electroporated, about 20% of the cells fluoresced singly or in clusters as intensely as those after WT transfection (Fig. 7D). In the medium, E1 and E2 glycoproteins were readily detected by Western blot analysis (data not shown). These results indicate that the 3V mutant and the 12V mutant could complement and replicate efficiently at 40°C. Since the 12V mutant can synthesize minus-strand RNA as efficiently as WT virus can at 40°C, factors required for plus-strand RNA synthesis must be supplied by the 3V mutant in which both the 1/2 and 2/3 sites are cleavable. The low efficiency of RNA transfection and the pronounced clustering of infected cells make it likely that the infection has spread from the initially transfected cells to neighboring cells.

DISCUSSION

Uncleaved P123 and nsP4 can transcribe minus-strand RNA whereas cleaved products from P123 are required for efficient plus-strand RNA synthesis. The fact that the 12V mutant could transcribe minus-strand RNA as efficiently as WT virus at early stages of infection indicates that uncleavable P123 and the mature nsP4 are sufficient for transcription of minus-strand RNA from the input viral RNA. At 30°C the amount of minus-strand RNA continued to increase up to 12 h postinfection, indicating that uncleaved P123 and the mature nsP4 can also synthesize plus-strand genomic and subgenomic RNA, albeit at a very low efficiency as shown by the very low incorporation of [³H]uridine. On the other hand, at 40°C the amount of minus-strand RNA did not increase after 2 h of incubation but, rather, decreased, indicating that the minus-strand RNA transcribed from the input RNA was not tran-

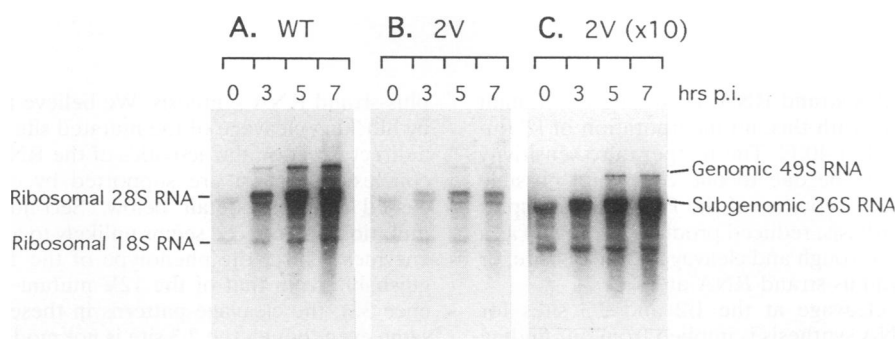


FIG. 6. Northern blot analysis of plus-strand RNA synthesis by WT virus and by the 2V mutant at 40°C. Cytoplasmic RNA was isolated from WT virus-infected chicken cells (A) or the 2V mutant-infected chicken cells (B and C) at 0, 3, 5, and 7 h after infection. [³²P]CTP-labeled in vitro RNA transcripts annealing to nucleotides 8571 to 10770 were used as the probe. For panels A and B, an X-ray film was exposed to the probed membranes for the same period. For panel C, an X-ray film was exposed for 10 times longer than for panels A and B to the same membrane used for panel B.

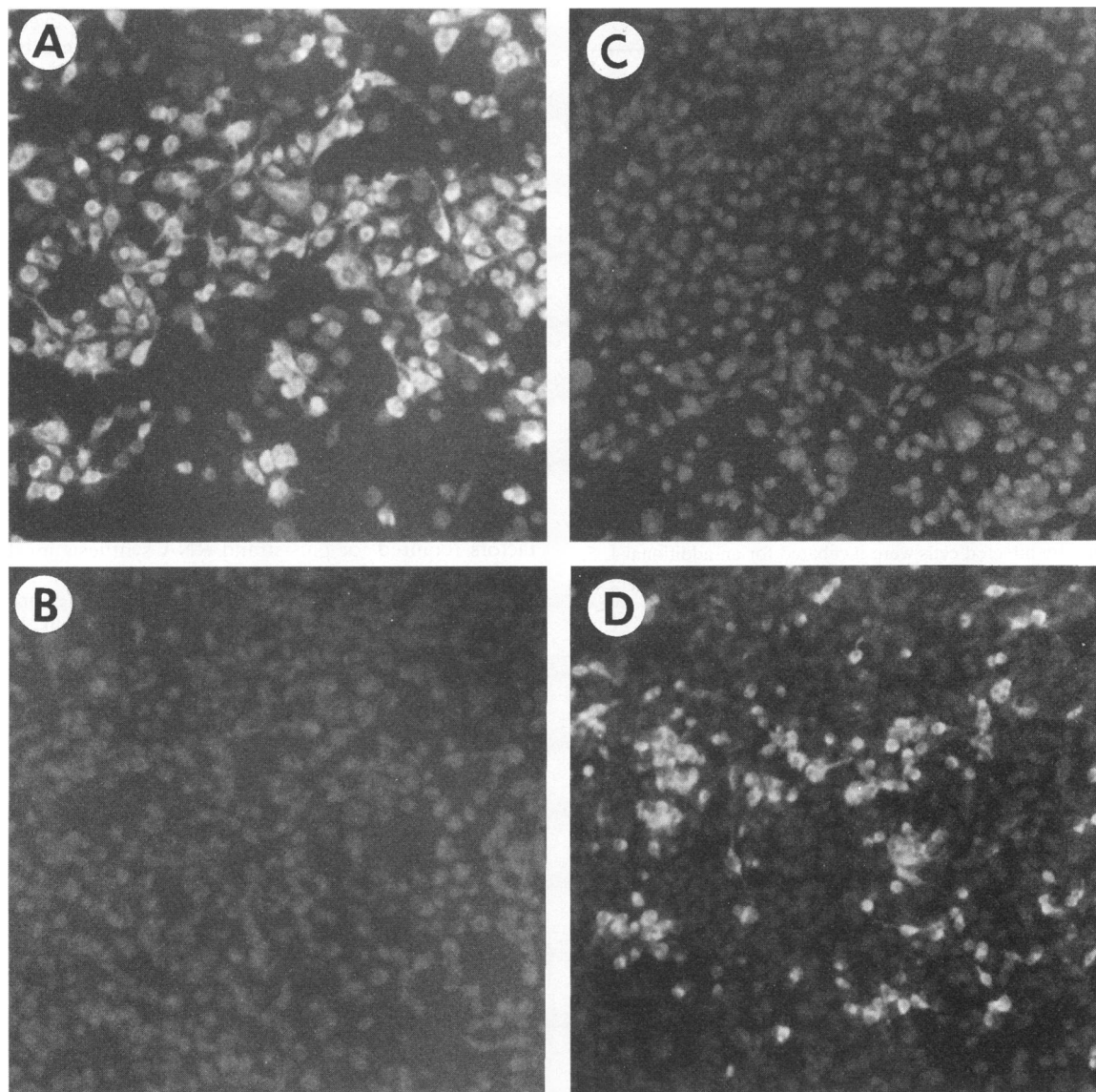


FIG. 7. Immunofluorescence assay of BHK-21 cells after electroporation with RNA transcripts from the WT, the 12V, and the 3V constructs. BHK-21 cells were transfected with WT transcripts (A), the 3V transcripts (B), the 12V transcripts (C), or a mixture of the 3V transcripts and the 12V transcripts (D) by using an electroporator. After electroporation, cells were incubated at 40°C for 15 h. Anti-E1 and anti-E2 rabbit polyclonal sera were used to probe the E1 and E2 glycoproteins, followed by a treatment with fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin and examination in a fluorescence microscope.

scribed into genomic plus-strand RNA, rendering the mutant nonviable. In agreement with this, no incorporation of [^3H]uridine could be detected at 40°C. The temperature sensitivity of the 12V mutant could be due to one or several possible factors, including reduced activities of the P123-nsP4 complex in plus-strand RNA synthesis, reduced production of nsP4 as a result of inefficient readthrough and cleavage at the 3/4 site, or decreased stability of minus-strand RNA at 40°C.

A requirement for cleavage at the 1/2 and 2/3 sites for efficient plus-strand RNA synthesis is implied from our finding that early minus-strand RNA synthesis by the 2V mutant and by the 12V mutant was as efficient as by WT virus but that late plus-strand RNA synthesis by these mutants was much reduced compared with that in WT virus. In these mutants the reduced and delayed virus growth presumably arises from inefficient

plus-strand RNA synthesis. We believe that the mutations act by blocking cleavage of the mutated site rather than by having a direct effect on the activities of the RNA replicase. First, the conclusions drawn are supported by other approaches discussed in more detail below. Second, the nature of the mutations introduced seems unlikely to affect the activity of the enzymes. Third, the phenotype of the 1V mutant is indistinguishable from that of the 12V mutant (this study and reference 32); the cleavage patterns in these two mutants are the same even though the 2/3 site is not modified in 1V, suggesting that it is the defect in cleavage that is important.

Lemm and Rice (19, 20) recently examined the activities of various forms and combinations of nonstructural proteins in RNA replication and transcription by using vaccinia virus expression systems. They showed that combinations of un-

cleavable P123 and cleavable P34 or N-terminally truncated forms of P34, which would produce authentic nsP4, can transcribe both minus-strand and plus-strand RNAs efficiently in BHK-21 cells at 37°C. They also found that P123 was required for RNA synthesis, because various combinations of intermediate and mature products did not result in the transcription of RNA even in the presence of authentic nsP4, although there is a possibility that the addition of Met at the N terminus of nsP2 and nsP3, required for their expression, made them nonfunctional.

The major difference between our results and those of Lemm and Rice (19, 20) is our finding of a requirement for cleavage of P123 for efficient plus-strand RNA synthesis. We propose that the efficient synthesis of plus-strand RNA by uncleavable P123 and nsP4 in the vaccinia virus system at 37°C arose from the overproduction and accumulation of the expressed proteins as well as from a consistent supply of plus-strand template RNA from a vaccinia virus construct, leading to continued production of minus-strand RNA in a high yield. During natural virus replication as used in our study, the numbers of template RNA and translated proteins are very limited at early stages of infection. In the 12V mutant-infected cells, a low activity of the P123-nsP4 complex in plus-strand RNA synthesis is probably a limiting factor for continuing synthesis of viral proteins and production of minus-strand templates, resulting in the inefficient production of both minus- and plus-strand RNAs.

The results of Strauss et al. (34) that a mutation in the proteinase that results in excessively rapid cleavage of P123 is lethal are also consistent with a requirement for uncleaved P123 in minus-strand RNA synthesis.

Our results that the nonviable mutant with the mutation in the 3/4 site can complement 12V suggest that production of nsP4 is essential for RNA replication. A direct effect of the mutation on the activity of the replicase cannot be ruled out, but the simplest interpretation is that cleavage at the 3/4 site is required for activity. Such a conclusion is supported by data of Lemm and Rice (20) and Lemm et al. (20a) that nsP4 produced either by cleavage at the 3/4 site or by cleavage of a ubiquitin-nsP4 construct is required for RNA synthesis in the vaccinia virus expression system.

Regulatory mechanisms in minus-strand and plus-strand RNA synthesis. The replication of alphavirus RNA consists of two distinct phases, as shown with both Sindbis virus and Semliki Forest virus (25, 28). Minus-strand RNA synthesis occurs only during the first 3 to 4 h after infection and then shuts down abruptly. Furthermore, minus-strand RNA synthesis requires continuous protein synthesis. In contrast, plus-strand genomic and subgenomic RNAs are synthesized throughout the infection cycle even if the protein synthesis is inhibited. It has also been shown that the proteolytic processing pathways of the nonstructural polyproteins also exhibit two phases (6, 32). Early in infection, when the numbers of genomic RNAs and translation products are limited, the 3/4 site in P1234 is cleaved rapidly in *cis*, whereas the 1/2 and 2/3 sites in P123 are cleaved only slowly because a *trans* cleavage at the 1/2 site is required to start the cleavage cascade (32). P123 and mature nsP4 are thus the major translation products at this stage. Later in infection, the concentration of the viral proteinase increases to the point at which the 2/3 site is cleaved rapidly, resulting in the production of P12 and nsP3 from P123 and of P12 and P34 from P1234; P12 is further cleaved into nsP1 and nsP2, whereas P34 accumulates as a result of the lack of active enzymes that cleave the 3/4 site (6, 14).

From the results of this study and those of previous studies, we propose that Sindbis virus RNA replication is regulated as

follows. Early in infection, mature nsP4 and uncleaved P123 are components of a minus-strand RNA replication complex. Since P123 is vulnerable to further processing, continued protein synthesis is required to supply P123 if minus-strand RNA synthesis is to continue. Processed products from P123 then form replication complexes for plus-strand RNA synthesis. Later in infection, when the protein concentration is increased, the 2/3 site is rapidly cleaved, probably while the polypeptide chains are still nascent. Under these conditions, P123 cannot exist or be produced, and thus minus-strand RNA synthesis is shut off. Since the plus-strand RNA replication complexes consist of already processed products, they are stable and can continue to synthesize plus-strand RNA even after protein synthesis is blocked. The change in template specificity of the WT replicase upon cleavage of P123 may arise from differences in conformation of the proteins induced by cleavage.

de Groot et al. (6) previously proposed that the differential processing pathway resulting from the different cleavage site specificities of the viral nonstructural proteinase resulted in the regulation of minus-strand RNA synthesis. Since the switch-over from slow processing of P123 to rapid processing coincided with the appearance of large amounts of P34, it was proposed that P34 played an active role in RNA synthesis (6, 13, 37). The results of this study, as well as those of Lemm and Rice (19, 20), of Sawicki and Sawicki (27), and of Barton et al. (2a), who showed that replication complexes isolated at 6 h after infection contained nsP1, nsP2, nsP3, and nsP4 and a cellular 120-kDa protein but little P34, suggest that P34 may arise simply as a by-product of the regulatory processing pathway, and it is not clear whether P34 plays any role in RNA synthesis.

The temperature-sensitive shutoff and reactivation of minus-strand RNA synthesis in *ts24*, revertants of *ts24* (*ts24R*), *ts17*, and *ts133* (26, 29, 30) may result from different mechanisms from those which regulate RNA synthesis during WT infection. Since these mutants do not require de novo protein synthesis for continuing or for resuming the minus-strand RNA synthesis at a nonpermissive temperature, replication complexes containing cleaved P123 products may be able to synthesize minus-strand RNA as well as plus-strand RNA in these mutants. Sawicki et al. (24) and Sawicki and Sawicki (27) suggested that the same replication complexes exchange templates between minus-strand RNA and plus-strand RNA and that both nsP2 and nsP4 function in the template recognition. It seems likely that changes in the conformation of nsP2 or nsP4 induced by these mutations allow the replication complexes to recognize not only minus-strand templates but also plus-strand templates and to initiate both plus-strand and minus-strand RNA synthesis. For the nsP2-containing proteinase mutants, it is possible that accumulation of uncleaved P123 as a result of the partial inactivation of nsP2-containing proteinase (13, 27) could also account in part for the continuing synthesis of minus-strand RNA at 40°C in experiments in which continuing protein synthesis is not blocked.

The model for regulation of RNA synthesis proposed here suggests that a function of the opal termination codon positioned near the end of nsP3 in most alphaviruses may be to accelerate the conversion of minus-strand replication complexes into plus-strand replication complexes. The low efficiency of readthrough of this termination codon, 5 to 20% depending on the conditions, means that the concentration of nsP2-containing proteinases builds up faster than the concentration of nsP4-containing replicases. This should result in an acceleration of the processing of minus-strand replicase com-

plexes containing P123 and nsP4 into plus-strand complexes containing the cleaved products.

Evolutionary relationship with Sindbis virus-like plant RNA viruses. nsP1, the N-terminal helicase domain of nsP2, and nsP4 of Sindbis virus share amino acid sequence homology with the corresponding domains of many plant RNA viruses having diverse morphology and genome organization (1, 9, 17). However, Sindbis virus-like plant RNA viruses differ from their animal virus counterparts in that their genomes do not encode a proteinase for maturation of the nonstructural proteins, with the exception of turnip yellow mosaic virus (a tymovirus), which appears to process a 206-kDa nonstructural protein (3). In terms of genome organization, Sindbis virus resembles the tobamoviruses, tobamoviruses, and soil-borne wheat mosaic furoviruses (33) in that the three conserved domains are placed in the same order as in the Sindbis virus genome and that the polymerase domain is located downstream of a partially suppressible termination codon. However, Sindbis virus differs from these plant viruses in that cleavage at the 3/4 site appears to be required for viability. The 123V and the P2C481G mutants of Sindbis virus make only the two overlapping proteins P123 and P1234 (Table 1), equivalent to the 126- and 183-kDa proteins of tobacco mosaic virus, respectively. These polyproteins are functional in tobacco mosaic virus but not in Sindbis virus. In this regard, Sindbis virus resembles hordeoviruses, bromoviruses, cucumoviruses, and ilarviruses, in which the polymerase domain is expressed separately from the other two domains. The 12V mutant produces uncleavable P123 and mature nsP4, which are equivalent to the 1a and 2a proteins of brome mosaic virus, respectively. Thus the mechanisms used by the Sindbis virus-like plant viruses to regulate minus-strand RNA synthesis must differ from those used by Sindbis virus, and comparison of these mechanisms will be of interest.

As shown from this study and previous studies, proteolytic processing of nonstructural protein(s) is vital for Sindbis virus RNA replication. During the course of evolution, a papain-like proteinase (15, 34) of cellular origin was probably incorporated into an ancestral RNA virus genome, which may have facilitated efficient RNA replication and perhaps allowed the virus to expand its host range to include both vertebrate and invertebrate hosts. If the ancestor to Sindbis virus was a plant virus or an invertebrate virus, the acquisition of a proteinase could have allowed the virus to efficiently regulate RNA replication at the higher body temperatures of birds and mammals.

ACKNOWLEDGMENTS

We thank Raoul de Groot for the gift of the 3/4 site mutants and Ellen Strauss for valuable comments on the manuscript.

This work was supported by grant AI10793 from the NIH.

REFERENCES

- Ahlquist, P., E. G. Strauss, C. M. Rice, J. H. Strauss, J. Haseloff, and D. Zimmermann. 1985. Sindbis virus proteins nsP1 and nsP2 contain homology to nonstructural proteins from several RNA plant viruses. *J. Virol.* **53**:536–542.
- Barton, D. J., S. G. Sawicki, and D. L. Sawicki. 1988. Demonstration in vitro of temperature-sensitive elongation of RNA in Sindbis virus mutant ts6. *J. Virol.* **62**:3597–3602.
- Barton, D. J., S. G. Sawicki, and D. L. Sawicki. Solubilization and immunoprecipitation of alphavirus replication complexes. *J. Virol.* **65**:1496–1506.
- Bransom, K. L., J. J. Weiland, and T. W. Dreher. 1991. Proteolytic maturation of the 206-kDa nonstructural protein encoded by turnip yellow mosaic virus RNA. *Virology* **184**:351–358.
- Burge, B. W., and E. R. Pfefferkorn. 1966. Isolation and characterization of conditional-lethal mutants of Sindbis virus. *Virology* **30**:204–213.
- Chamberlain, J. P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. *Anal. Biochem.* **98**:132–135.
- de Groot, R. J. Unpublished data.
- de Groot, R. J., W. R. Hardy, Y. Shirako, and J. H. Strauss. 1990. Cleavage-site preferences of Sindbis virus polyproteins containing the non-structural proteinase. Evidence for temporal regulation of polyprotein processing *in vivo*. *EMBO J.* **9**:2631–2638.
- de Groot, R. J., T. Rumenapf, R. J. Kuhn, E. G. Strauss, and J. H. Strauss. 1991. Sindbis virus RNA polymerase is degraded by the N-end rule pathway. *Proc. Natl. Acad. Sci. USA* **88**:8967–8971.
- Ding, M., and M. J. Schlesinger. 1989. Evidence that Sindbis virus nsP2 is an autoprotease which processes the virus nonstructural polyprotein. *Virology* **171**:280–284.
- Goldbach, R., O. L. Gall, and J. Wellink. 1991. Alpha-like viruses in plants. *Semin. Virol.* **2**:19–25.
- Gorbalenya, A. E., V. M. Blinov, A. P. Donchenko, and E. V. Koonin. 1989. An NTP-binding motif is the most conserved sequence in a highly diverged monophyletic group of proteins involved in positive strand RNA viral replication. *J. Mol. Evol.* **28**:256–268.
- Hahn, Y. S., A. Grakoui, C. M. Rice, E. G. Strauss, and J. H. Strauss. 1989. Mapping of RNA⁺ temperature-sensitive mutants of Sindbis virus: complementation group F mutants have lesions in nsP4. *J. Virol.* **63**:1194–1202.
- Hahn, Y. S., E. G. Strauss, and J. H. Strauss. 1989. Mapping of RNA⁺ temperature-sensitive mutants of Sindbis virus: assignment of complementation groups A, B, and G to nonstructural proteins. *J. Virol.* **63**:3142–3150.
- Hardy, W. R., Y. S. Hahn, R. J. de Groot, E. G. Strauss, and J. H. Strauss. 1990. Synthesis and processing of the nonstructural polyproteins of several temperature-sensitive mutants of Sindbis virus. *Virology* **177**:199–208.
- Hardy, W. R., and J. H. Strauss. 1988. Processing of the nonstructural polyproteins of Sindbis virus: study of the kinetics *in vivo* by using monospecific antibodies. *J. Virol.* **62**:998–1007.
- Hardy, W. R., and J. H. Strauss. 1989. Processing the nonstructural polyproteins of Sindbis virus: nonstructural proteinase is in the C-terminal half of nsP2 and functions both *in cis* and *in trans*. *J. Virol.* **63**:4653–4664.
- Kamer, G., and P. Argos. 1984. Primary structural comparison of RNA-dependent polymerases from plant, animal and bacterial viruses. *Nucleic Acids Res.* **12**:7269–7282.
- Koonin, E. V. 1991. The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. *J. Gen. Virol.* **72**:2197–2206.
- Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* **82**:488–492.
- Lemm, J. A., and C. M. Rice. 1993. Assembly of functional Sindbis virus RNA replication complexes: requirement for coexpression of P123 and P34. *J. Virol.* **67**:1905–1915.
- Lemm, J. A., and C. M. Rice. 1993. Roles of nonstructural polyproteins and cleavage products in regulating Sindbis virus RNA replication and transcription. *J. Virol.* **67**:1916–1926.
- Lemm, J. A., T. Rumenapf, E. G. Strauss, J. H. Strauss, and C. M. Rice. 1994. Unpublished data.
- Li, G., and C. M. Rice. 1989. Mutagenesis of the in-frame opal termination codon preceding nsP4 of Sindbis virus: studies of translational readthrough and its effect on virus replication. *J. Virol.* **63**:1326–1337.
- Mi, S., R. Durbin, H. V. Huang, C. M. Rice, and V. Stollar. 1989. Association of the Sindbis virus RNA methyltransferase activity with the nonstructural protein nsP1. *Virology* **170**:385–391.
- Rice, C. M., R. Levis, J. H. Strauss, and H. V. Huang. 1987. Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and *in vitro* mutagenesis to generate defined mutants. *J. Virol.* **61**:3809–3819.
- Sawicki, D. L., D. B. Barkhimer, S. G. Sawicki, C. M. Rice, and S. Schlesinger. 1990. Temperature sensitive shut-off of alphavirus

- minus strand RNA synthesis maps to a nonstructural protein, nsP4. *Virology* **174**:43–52.
25. Sawicki, D. L., and S. G. Sawicki. 1980. Short-lived minus-strand polymerase for Semliki Forest virus. *J. Virol.* **34**:108–118.
 26. Sawicki, D. L., and S. G. Sawicki. 1985. Functional analysis of the A complementation group mutants of Sindbis HR virus. *Virology* **144**:20–34.
 27. Sawicki, D. L., and S. G. Sawicki. 1993. A second nonstructural protein functions in the regulation of alphavirus negative-strand RNA synthesis. *J. Virol.* **67**:3605–3610.
 28. Sawicki, D. L., S. G. Sawicki, S. Keranen, and L. Kaariainen. 1981. Specific Sindbis virus-coded function for minus-strand RNA synthesis. *J. Virol.* **39**:348–358.
 29. Sawicki, S. G., and D. L. Sawicki. 1986. The effect of loss of regulation of minus-strand RNA synthesis on Sindbis virus replication. *Virology* **151**:339–349.
 30. Sawicki, S. G., D. L. Sawicki, L. Kaariainen, and S. Keranen. 1981. A Sindbis virus mutant temperature-sensitive in the regulation of minus-strand RNA synthesis. *Virology* **115**:161–172.
 31. Scheidel, L. M., and V. Stollar. 1991. Mutations that confer resistance to mycophenolic acid and ribavirin on Sindbis virus map to the nonstructural protein nsP1. *Virology* **181**:490–499.
 32. Shirako, Y., and J. H. Strauss. 1990. Cleavage between nsP1 and nsP2 initiates the processing pathway of Sindbis virus nonstructural polyprotein P123. *Virology* **177**:54–64.
 33. Shirako, Y., and T. M. A. Wilson. 1993. Complete nucleotide sequence and organization of the bipartite RNA genome of soil-borne wheat mosaic virus. *Virology* **195**:16–32.
 34. Strauss, E. G., R. J. de Groot, R. Levinson, and J. H. Strauss. 1992. Identification of the active site residues in the nsP2 proteinase of Sindbis virus. *Virology* **191**:932–940.
 35. Strauss, E. G., C. M. Rice, and J. H. Strauss. 1984. Complete nucleotide sequence of the genomic RNA of Sindbis virus. *Virology* **133**:92–110.
 36. Strauss, E. G., and J. H. Strauss. 1986. Structure and replication of the alphavirus genome, p. 35–90. *In* S. Schlesinger and M. J. Schlesinger (ed.), *The Togaviridae and Flaviviridae*. Plenum Press, New York.
 37. Strauss, J. H., and E. G. Strauss. 1990. Alphavirus proteinases. *Semin. Virol.* **1**:347–356.
 38. Wang, Y.-F., S. G. Sawicki, and D. L. Sawicki. 1991. Sindbis virus nsP1 functions in negative-strand RNA synthesis. *J. Virol.* **65**:985–988.